The advantages of cDNA microarray as an effective tool for identification of reproductive organ-specific genes in a model legume, *Lotus japonicus*

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Abstract To understand the molecular mechanisms intrinsic to reproductive organ development a cDNA microarray, fabricated from flower bud cDNA clones, was used to isolate genes, which are specifically expressed during the development of the anther and pistil in *Lotus japonicus*. Cluster analysis of the microarray data revealed 21 and 111 independent cDNA groups, which were specifically expressed in immature and mature anthers, respectively. RT-PCR was performed to provide a direct assessment of the accuracy and reproducibility of our approach. Confirmation of our results suggests that cDNA microarray technology is an effective tool for identification of novel reproductive organ-specific genes.

Key words: cDNA microarray; Anther-specific gene; Pistil-specific gene; Reverse transcriptase polymerase chain reaction; *Lotus japonicus*

1. Introduction

Most species of angiosperms produce hermaphroditic flowers that are comprised of both male and female reproductive organs. The developmental programs of these male and female organs are substantially independent of each other and of the vegetative plant. It is of great interest to discover the underlying molecular mechanisms that control the development of the stamen and pistil and the interaction between the pollen and pistil. Analysis of the molecular mechanism controlling the development and differentiation of reproductive organs is immensely important since knowledge of gene regulation during sexual reproduction of the flowering plant may result in potential applications in agriculture such as hybrid seed production by using male sterility.

To date, two major strategies have been utilized to isolate and characterize genes involved in sexual reproduction. One strategy involves the isolation of genes associated with previously characterized genetic loci, including the self-incompatibility (SI) response and cytoplasmic male sterility (CMS). In the case of SI in *Brassica* species, these genes (*SLG*, *SRK* and *SP11*/*SCR*) regulate self-pollen recognition, termed *S* locus (reviewed in [1]). The CMS-associated region in the mitochondrial genome and a restoration gene of the CMS in the nuclear genome have also been isolated and characterized through dissection of this genetic locus (reviewed in [2]).

Genes involved in sexual reproduction have also been isolated through spatial and temporal gene expression studies that monitor genes which are specifically or predominantly expressed in reproductive organs followed by functional characterization of these genes from the viewpoint of sexual reproduction. Isolation of these genes by differential expression in the reproductive organs was usually identified by subtractive hybridization, differential plaque hybridization, differential display or protein gel differences, followed by micro-sequencing or using antibodies to cDNA clones. As a result, there have been many reports describing genes from various plant species specifically expressed in anthers, pollen, or pistils (reviewed in [3,4]). However, not all members of the reproductive organ-specific genes have been identified and characterized, because it is estimated that a pollen grain contains about 2000 specifically or dominantly expressed genes [5]. Therefore, to identify novel reproductive organ-specific genes, we utilized a cDNA microarray for an extensive, sensitive, and simultaneous survey of genes involved in floral reproductive organ development.

Microarrays, which are made from cDNA spotted onto a glass slide at a high density, are widely recognized as a significant technological advance, which provide genome-scale information about gene expression patterns [6,7]. Microarrays are hybridized simultaneously with two sets of color fluorescently labeled cDNA that are prepared from RNA samples of different cells or organs treated with stress. In plant science, the majority of microarray studies involve the identification and characterization of genes which respond to several kinds of stress (salt, drought, cold stress, wounding, insect feeding, pathogen infection, etc. [8–11]). In addition, a few papers report the use of microarray technology for analysis of developmentally regulated genes in higher plants [12,13]. However,

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there are no reports concerning the analysis of genes related to the development and differentiation of reproductive organs with DNA microarray technology in plants.

Lotus japonicus has emerged as a model legume for genome analysis, due to the small size of its genome, its short generation time, and self-compatibility [14]. Recently, a large number of expressed sequence tags (ESTs) have been generated from seedlings, roots and flower buds in *L. japonicus* [15, 16], providing sequence information for many transcripts of *L. japonicus*. Additionally, the size of *L. japonicus* flowers is much larger than in other model plant species, such as *Arabidopsis* and rice, thus facilitating the dissection of gene expression in floral reproductive organs.

In the present study, microarray elements derived from a cDNA library of flower buds of *L. japonicus* were assembled to identify the reproductive organ-specific transcripts compared with those of the leaf tissues. Cluster analysis showed that over 100 anther- or pistil-specific genes were identified. In reverse transcriptase polymerase chain reaction (RT-PCR) analysis, confirmed anther-specific genes showed the similar expression pattern as determined by a DNA microarray experiment.

2. Materials and methods

2.1. Plant materials and mRNA isolation

Plants of *L. japonicus* Gifu B-129 were grown in a greenhouse. Flower buds were classified into three stages according to bud length. Flower buds in stage 1 were 5–6 mm in length, and contained uninucleate microspores. Flower buds in stage 2 were 8–9 mm in length, and contained uninucleate microspores and binucleate pollen grains. Flower buds in stage 3 were more than 10 mm in length, just before anther dehiscence. Flower buds in stages 1 and 3 were collected for constructing the cDNA library. Anther and pistil tissues in each stage were also collected for expression analysis as described below. Leaf tissues were harvested during reproductive growth.

Total RNA was isolated from buds, anther, pistil and leaf tissues as described previously [16]. Isolation of poly(A)$^+$ RNA from the total RNA was performed using a FastTrack 2.0 mRNA isolation kit (Invitrogen, San Diego, CA, USA).

2.2. cDNA library construction

Two cDNA libraries were constructed from flower buds. One library was constructed from flower buds of stage 1. The other was constructed from flower buds of stage 3 as described in [16]. Total RNA and mRNA were isolated from flower buds as described above. Double-stranded cDNA was synthesized using a λZAPII cDNA synthesis kit (Stratagene, La Jolla, CA, USA). The cDNA was ligated to a Uni-ZAP XR vector (Stratagene), and the ligated cDNA was packaged in vitro using a Gigapack extract (Stratagene). The phage library was converted to plasmid form by mass excision according to the procedure described by Stratagene. Plasmids containing inserts of more than 500 bp were selected from the library by using PCR with a M13 universal primer set.

2.3. Preparation of the cDNA microarray

1760 and 2288 clones were picked at random from the cDNA library of flower buds in stage 1 and stage 3, respectively. 898 out of 2288 clones derived from stage 3 were partially sequenced from the 5′-end [16]. After the plasmid DNA was isolated, insert cDNA was amplified by PCR using a M13 universal primer set. PCR products were purified using QIAquick 96-column (Qiagen, Basel, Switzerland) and Multiscreen PCR (Millipore, Benford, MA, USA). The purified cDNA insert was mixed with reagent D (Amersham Pharmacia, Uppsala, Sweden), and each cDNA was spotted in duplication on aluminum-coated and DMSO-optimized glass slides using an Array Spotter Generation III (Amersham Pharmacia).

2.4. Fluorescent probe preparation, hybridization and scanning

Labeling of each poly(A)$^+$ RNA with Cy3-dUTP or Cy5-dUTP and hybridization was performed as previously described [17]. Microarrays were scanned in both the Cy3 and Cy5 channels with a GenePix 4000A Microarray scanner (Axon Instruments, Foster City, CA, USA).

2.5. Data analysis

The fluorescence intensity for each fluor and each element was captured by using ArrayGauge (Fuji Film, Tokyo, Japan). The local background was subtracted from the value of each spot on the array. Normalization of Cy3 and Cy5 signal intensity was performed by adjusting the total signal intensities of the two images. This operation was termed 'global normalization'. Signal data of each element in Cy3 and Cy5 were calculated according to following formula: the value of signal data = (signal intensity of each element)/(total signal intensities) $\times 10^6$. This value of calculated signal data for each element was used for further analysis.

2.6. Sequence analysis

The partial sequences from the 5′-end of 898 EST clones, derived from the cDNA library of a flower bud in stage 3, were previously determined [16]. The sequence of the cDNA library of the flower bud in stage 1 was completed from the partially sequenced 5′-end of 960 clones (M. Endo and M. Watanabe, unpublished data). cDNA clones which were characterized as anther- and/or pistil-specific clones by cDNA microarray analysis were sequenced by using the dideoxy chain termination method using a model 310 DNA sequencer (PE Biosystems, Foster City, CA, USA). In order to identify the number of independent anther- and/or pistil-specific clones, clustering of the EST sequences was performed. The 5′-end sequence was compared with a dataset of itself using the BLAST program [18], and the sequences that showed over 95% identity for more than 100 bp were grouped together. Homology searches of each specific clone which was grouped were performed using the BLAST program [18] available at the website of the National Institute of Genetics (http://ddbj.nig.ac.jp). Similarity was considered to be significant when the optimized similarity BLAST score was higher than 100 at the amino acid level.

2.7. RT-PCR

RT-PCR was performed according to [19]. Briefly, poly(A)$^+$ RNA was isolated from anthers of stages 1–3 and pistils of stage 3, and leaves were reverse-transcribed to synthesize first strand cDNA by using the First-Strand cDNA synthesis kit (Amersham-Pharmacia). Then cDNA was used as a template for PCR amplification with a set consisting of primer *Not*I-dT (5′-AACTGGAAGAATTCGCGGCCGCAGGGAAT-3′) and a primer specific to each gene. PCR was performed with *ExTaq* DNA polymerase (TaKaRa Shuzo, Shiga, Japan) for 25 or 30 cycles of denaturation for 1 min at 95°C, annealing for 2 min at 60°C and extension for 2 min at 72°C, followed by a final extension for 5 min.

3. Results and discussion

3.1. Strategy for expression analysis with cDNA microarray

To analyze anther- and/or pistil-specific genes during reproductive organ development both qualitatively and quantitatively in *L. japonicus*, we used the microarray technique to monitor gene expression patterns of 4048 clones. To increase the reliability of the detected signals, each PCR sample was spotted twice in subarrays resulting in total 8096 data points. When each sample was reverse-transcribed with fluorescent dye, mRNA derived from anther or pistil tissues in different developmental stages (stages 1–3, see Section 2) was labeled with Cy5. In contrast, mRNA from leaf tissues, a vegetative organ, was labeled with Cy3. The samples were labeled with different dyes, then mixed and hybridized simultaneously to a cDNA microarray. After scanning the Cy5 and Cy3 channels and global normalization, the Cy5/Cy3 ratio was calculated using the global normalized data in each element. Because the elements (right and left positions) were spotted twice for each clone as described above, comparison of the Cy5/Cy3 ratio

Table 1
The number of genes abundantly expressed in anther and pistil tissue in each developmental stage

Signal ratio of reproductive organ to leaf	Anther			Pistil		
	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
6.0 < Cy5/Cy3 < 10	53	68	48	24	66	55
10 < Cy5/Cy3	24	110	106	2	17	23

between the right and left duplicated elements was performed. In each clone, when the Cy5/Cy3 ratio of the right position to the left position ([Cy5/Cy3 ratio of right position]/[Cy5/Cy3 ratio of left position]) ranged from 1/3 to 3, the data of the clones were adopted, and the Cy5/Cy3 ratio data averaged between the right and the left on the glass was used for further analysis. In contrast, when the Cy5/Cy3 ratio of right to left was under 1/3 or over 3, the data of the clones were eliminated, and attributed to experimental error.

To assess the reproducibility of the microarray analysis, each tissue comparison was performed four or five times using independently isolated RNA samples as the starting material. Comparison of the Cy5/Cy3 ratio among the different slide glasses (four or five experiments) in each clone was performed. Following the comparison, when the Cy5/Cy3 data which ranged from 1/3 to 3 were observed over three times, the adopted Cy5/Cy3 ratio was averaged in each clone. As a result, about 80% of 4048 clones finally were utilized in each experiment.

3.2. Identification of genes abundantly expressed in anther and/or pistil tissues

In order to identify the abundantly expressed genes in anther and/or pistil tissues, the number of clones whose Cy5/Cy3 ratio was over six-fold or 10-fold was counted in all six experimental combinations (three stages of anther and three stages of pistil versus one stage of leaf tissue), as shown in Table 1. In the anther versus leaf co-hybridizations, approximately 7% (281 clones) of the 4048 clones showed more than six-fold stronger signals in each of the stages of the anthers, and approximately 4% (160 clones) were expressed more than 10-fold higher in each of the stages of the anthers than in that of the leaves. In the case of co-hybridization with cDNAs derived from mRNAs of pistil and leaf tissues, similar comparisons yielded 3% (124 clones) and 1% (40 clones) of the analyzed genes, respectively. The hybridization kinetics experiments with mRNA from pollen grains and vegetative shoots demonstrated that approximately 10% of genes expressed in pollen grain might be unique in *Tradescantia* and *Zea* [5,20]. In our experiments, the percentage of clones whose Cy5/Cy3 ratio was over six-fold was about 7% in anther tissues. Thus, in this study, the genes that are abundantly expressed in anther and/or pistil tissues are defined as clones with a minimum of six-fold up-regulation in their transcript level against the control, leaf tissues. During the developmental stage of anther, the number of highly expressed genes in anther tissues increased. A similar trend was observed in pistil (Table 1).

BLAST analysis of the 281 cDNA clones identified as highly expressed in anther tissues generated 204 non-redundant groups. In the case of the highly expressed cDNA clones in the pistil tissues, 124 of them were classified into 64 non-redundant groups using BLAST analysis. In the course of grouping the clones, the highly redundant cDNA clones expressed in anther showed strong signal intensity in anthers (Table 2). Because cDNAs on the microarray were randomly chosen, it is highly probable that frequently redundant clones showed high signal intensity in the anther.

3.3. Cluster analysis of cDNA highly expressed in anther tissues

In order to identify cDNA clones specifically expressed in anther tissues, and to detect the cDNA clones which were co-regulated according to the developmental stage in anther tissues, cluster analysis of highly expressed clones in anther tissues (anthers/leaves > 6.0) was performed using a Cluster and Tree viewer [21]. For construction of a hierarchical cluster tree, we used the signal data averaged for each stage of the samples (anthers 1–3, pistils 1–3, and leaves), instead of the Cy5 (signal from reproductive tissues)/Cy3 (signal from leaf tissues) ratio, for the purpose of identifying anther-specific genes that are not expressed or are expressed at an extremely low level in other pistil and leaf tissues.

Cluster analysis revealed several groups of cDNA clones with similar behavior during anther development (Fig. 1). Two distinct patterns emerged from the cluster analysis. At first, the cDNA clones which were contained in cluster I (this cluster will be referred to as cluster I-a hereafter) were highly expressed in the stage 1 anther tissues, and slightly expressed in other stages and tissues. Secondly, the clones which were contained in cluster II (this cluster will be referred to as cluster II-a hereafter) are highly expressed in stages 2 and 3 anther tissues, and slightly expressed in other stages and tissues (Fig. 1, Table 2). Twenty-one cDNA clones out of 24, which were contained in cluster I-a, were derived from the flower bud cDNA library in stage 1. In contrast, 152 cDNA clones out of 159, which were contained in cluster II-a, were derived from the flower bud cDNA library in stage 3, indicating that it was important to construct a specialized cDNA library for identification of organ-specific genes. Cluster I-a and cluster II-a contained 21 non-redundant groups (24 clones) and 111 non-redundant groups (159 clones), respectively. In contrast, we could not determine whether the clones which were classified into the different groups though they were annotated to the same functional genes were derived from the same genes or from redundant homologous genes.

The reliability of our experiment was confirmed by two independent viewpoints. One is that each clone was spotted on the glass slide plate in duplicate. The other viewpoint is that the clones which were classified into the same group showed a similar trend in their temporal expression pattern. In both clusters I-a and II-a, there were several clones which have already been characterized as pollen- and/or anther-specific genes, as described below. Overall reliability of our microarray was confirmed by obtaining the expected preferential anther or pollen expression patterns for dozens of well-known characterized genes (Table 2).

Fourteen groups (17 clones) out of 21 (24 clones) and 63 groups (84 clones) out of 111 (159 clones), which were contained in clusters I-a and II-a respectively, showed no signifi-

Table 2
List of anther- or pistil-specific genes obtained from cluster analysis

Group[a]	No. of clones[b]	Homologous gene[c] (accession no.)	Expression level[d]						
			Anther			Pistil			Leaf
			Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	
Cluster I-a									
Group A-28	1	Histone deacetylase HD2 (AF255711-1)	2877	28.16	N.D.[e]	91.7	57.8	46.4	35.5
Group A-50	1	Allyl alcohol dehydrogenase (AB036735-1)	369.3	56.09	40.33	49.0	30.8	41.2	41.6
Group A-76	1	CER1 (AC026815-18)	509.9	121.6	103.2	109.5	71.4	114.9	58.4
Group A-125	1	Δ^1-pyrroline-5-carboxylate synthetase (U92286-1)	872.2	334.2	304.7	61.8	49.3	61.2	54.1
Group A-176	1	Δ^1-pyrroline-5-carboxylate synthetase (U60267-1)	510	220.1	107.2	27.2	37.4	37.6	29.2
Group A-191	1	Cytochrome P450 (AB024931-1)	736.6	74.7	46.66	39.0	68.3	40.9	158.4
Group A-204	1	Cytosolic glutamine synthetase (U15591-1)	1091	453.7	458.3	211.2	202.8	177.7	140.2
Cluster II-a									
Group A-1	7	Pectin methylesterase (Y13285-1)	82.1	4489.0	4342.9	87.0	109.0	76.6	67.4
Group A-18	2	Pectin methylesterase (AB029461-1)	29.2	257.2	362.5	40.1	46.2	39.8	41.6
Group A-13	3	Pectin esterase (AP000603-3)	66.5	2014.9	2007.3	22.1	30.9	21.0	65.5
Group A-8	5	Ascorbate oxidase (S24950)	72.5	3407.3	3082.1	35.3	46.2	24.4	43.3
Group A-146	1	Ascorbate oxidase (X61146-1)	84.0	3540.7	3874.2	15.2	N.D.	N.D.	35.9
Group A-26	2	L-Ascorbate oxidase (AP000603-2)	85.9	4005.1	4298.6	10.0	62.5	49.8	53.4
Group A-150	1	L-Ascorbate oxidase (X61146-1)	100.6	2050.7	2119.1	40.3	66.3	36.8	85.6
Group A-20	2	β-Galactosidase (AC005825-14)	39.7	657.2	587.0	36.4	51.9	28.8	57.9
Group A-112	1	β-Galactosidase (AJ250431-1)	88.7	N.D.	3335.5	56.7	80.6	60.7	63.7
Group A-87	1	β-Galactosidase (AJ270310-1)	51.1	2613.3	2012.7	N.D.	23.6	14.4	46.8
Group A-84	1	β-Galactosidase (AC005825-14)	8.7	1570.5	1083.1	N.D.	27.7	N.D.	31.6
Group A-99	1	Polygalacturonase (U20431)	19.1	186.8	340.0	25.2	24.8	25.7	40.3
Group A-12	4	Actin (U81049-1)	288.3	1254.8	1069.5	214.0	243.3	232.4	138.0
Group A-128	1	Actin (U81049-1)	179.3	1141.1	829.6	154.0	443.2	244.8	192.5
Group A-25	2	Actin (U27981-1)	118.4	616.7	436.1	113.5	117.9	137.4	74.7
Group A-165	1	Actin (U27981-1)	280.1	1063.9	972.9	211.4	191.6	258.7	92.1
Group A-144	1	Actin (U68462-1)	104.8	501.0	465.6	102.7	77.9	126.9	59.3
Group A-93	1	Actin-depolymerizing factor (S30935)	39.5	509.6	341.3	17	17.9	12.6	20.0
Group A-3	6	Monosaccharide transporter (AB007648-13)	61.2	2204.5	2524.2	25.7	43.3	18.7	56.1
Group A-184	1	Sucrose transporter (AF176950-1)	140.2	610.6	454.5	42.8	48.9	32.4	53.6
Group A-124	1	Glucose transporter (AL161555-11)	82.3	4800.8	4859.5	26.3	55.9	N.D.	44.1
Group A-81	1	Cell wall invertase (Z35162-1)	108.0	375	316.3	45.6	N.D.	N.D.	41.4
Group A-24	2	Methionine synthase (AF214735-1)	165.0	1019.6	746.1	151.5	205.0	279.4	106.9
Group A-110	1	Methionine synthase (AC009540-17)	358.6	2578.5	1641.9	447.6	414.2	724.1	262.1
Group A-132	1	Methionine synthase (AC009540-17)	400.6	1352.3	1381.8	241.1	207.7	351.0	157.0
Group A-151	1	*S*-Adenosyl-L-methionine synthetase (Z24743-1)	138.5	541.6	466.2	84.8	82.4	65.4	41.8
Group A-52	1	Cobalamin-independent methionine synthase (U97200-1)	57.4	654.8	555.5	N.D.	N.D.	N.D.	38.3
Group A-29	2	Plasma membrane proton ATPase (AB022442-1)	112.4	377.2	322.5	24.0	N.D.	18.2	39.8
Group A-154	1	Plasma membrane proton ATPase (AJ295612-1)	53.2	1470	1644.1	25.6	N.D.	N.D.	57.0
Group A-153	1	Plasma membrane proton ATPase (AL138640-9)	158.2	1708.7	1546.6	32.4	40.9	32.7	75.3
Group A-139	1	Plasma membrane ATPase (X85804-1)	50.8	2003.3	1331.2	N.D.	N.D.	N.D.	67.2
Group A-131	1	Receptor-like protein kinase (U58474-1)	15.6	335.8	238.3	45.4	35.2	36.5	48.4
Group A-121	1	Pollen allergen (*Ole e 1*) (AL161549-4)	26.0	1848.8	1414.2	19.8	15.6	N.D.	35.5
Group A-120	1	Metallothionein-II (AF004808-1)	211.7	N.D.	725.9	N.D.	38.8	N.D.	43.0
Group A-113	1	*S*-Adenosyl-L-homocysteine hydrolase (Z26881-1)	199.1	1468.8	1210.5	226.0	198.5	301.9	146.5
Group A-102	1	Gip1 protein (S54832)	40.2	1341.8	976.6	106.2	143.7	224.8	93.1
Group A-75	1	Cadinene synthase (Q43714)	9.2	690.4	529.6	23.1	N.D.	N.D.	38.7
Group A-74	1	Cytochrome *c* (P00051)	130.1	509.5	313.0	89.5	119.5	118.2	75.1
Group A-71	1	γ-Tocopherol methyltransferase (AF104220-1)	98.9	6794.9	6379.2	N.D.	73.2	38.6	40.3
Group A-55	1	RUVB-like protein (AJ276264-1)	123.3	2490.9	2003.4	89.3	41.1	50.8	118.4
Group A-51	1	Lipoxygenase (AJ276265-1)	21.5	1230.7	1151.0	43.1	22.7	N.D.	46.5
Group A-48	1	40S ribosomal protein (AF067732-1)	44.4	1549.6	1101.3	7.4	21.6	N.D.	39.8
Group A-19	1	Peroxyredoxin (AF133302-1)	149.0	392.1	430.2	96.8	100.3	96.3	83.9
Group A-195	1	Reversibly glycosylated polypeptide-2 protein (AL391144-3)	110.8	1710.5	1101.5	196.2	225.4	191.7	93.6
Group A-47	1	DNA binding protein ACBF (AC007296-20)	82.2	4366.3	4000.6	19.8	54.8	26.2	48.5
Group A-45	1	Acid phosphatase (AJ223074-1)	19.8	757.0	470.8	N.D.	N.D.	12.6	98.8
Group A-35	1	3-Ketoacyl-CoA thiolase B protein (X75329-1)	101.3	392.5	244.7	N.D.	88.8	106.3	39.0
Group A-34	1	NADPH-dependent mannose 6-phosphate reductase (AF055910-1)	124.0	486.7	338.1	76.2	N.D.	73.6	44.4
Cluster I-p									
Group P-53	1	α-Xylosidase (AJ131520-1)	310.1	90.1	54.7	855.6	658.1	799.1	224.8
Group P-34	1	Anthocyanidin hydroxylase (X71360-1)	52.3	43.9	94.0	298.2	361.2	369.1	76.43
Group P-49	1	ATP-dependent transmembrane transporter (AC024261-11)	236.6	97.2	127.7	659.3	1430.4	968.4	207.3
Group P-4	5	Chalcone synthase (AF026258-1)	319.9	31.8	16.6	656.7	666.9	1006.9	180.2

Table 2 (*Continued*)
List of anther- or pistil-specific genes obtained from cluster analysis

Group[a]	No. of clones[b]	Homologous gene[c] (accession no.)	Expression level[d]						
			Anther			Pistil			Leaf
			Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3	
Group P-6	4	Chalcone synthase (D10223-1)	446.4	37.6	42.8	1394.4	1389.3	1887.4	328.1
Group P-30	1	Chalcone synthase (AJ006780-1)	41.3	33.3	21.5	563.9	771.8	1022.4	94.41
Group P-40	1	Chalcone synthase (D10223-1)	584.4	53.4	39.9	1662.0	1497.9	2498.7	431.3
Group P-50	1	Flavonone-3-hydroxylase (U33932-1)	158.6	76.1	45.3	1944.6	1682.9	2731.8	339.6
Group P-8	2	Flavonone-3-hydroxylase (X81812-1)	209.2	44.7	27.5	2046.2	2108.5	2528.1	361.5
Group P-5	3	GDSL-motif lipase/acylhydrolase (AC016829-9)	58.4	62.3	120.5	334.4	643.3	186.3	74.1
Group P-45	1	GDSL-motif lipase/acylhydrolase (AC016829-9)	29.0	89.5	55.0	244.8	317.8	147.8	61.43
Group P-55	1	GDSL-motif lipase/acylhydrolase (AC016829-9)	28.8	39.5	72.8	248.0	391.2	146.1	40.47
Group P-2	11	Lipid transfer protein (AJ002958-1)	63.4	52.5	60.7	870.4	1404.6	1461.6	120.4
Group P-7	3	Lipid transfer protein (AJ002958-1)	42.2	29.7	19.6	480.2	729.8	899.6	85.8
Group P-15	1	Lipoxygenase (X76124-1)	349.4	110.9	80.0	3245.9	2736.0	1863.5	
	1075								
Group P-23	1	Lipoxygenase (AJ276265-1)	405.1	70.1	36.5	1797.5	1604.0	906.0	447.1
Group P-48	1	Lipoxygenase (AJ276265-1)	89.7	25.8	18.8	870.3	572.6	518.0	138.1
Group P-47	1	Nod factor binding lectin-nucleotide phosphohydrolase (AF156780-1)	58.3	22.0	14.1	475.3	289.8	391.8	67.32
Group P-61	1	Oligopeptidase (AF078916-1)	88.1	81.8	78.3	378.9	322.1	631.6	63.56
Group P-44	1	Polygalacturonase inhibitor-like protein (AB025629-13)	13.4	14.6	15.7	32.0	96.1	83.1	35.6
Group P-9	2	Protease inhibitor (U12150-1)	202.5	92.7	74.7	1211.4	995.3	1135.4	148.1
Group P-39	1	Ribitol dehydrogenase-like protein (AB026650-14)	62.4	58.7	52.1	682.3	900.4	1073.7	118.3
Group P-63	1	Vacuolar ATPase subunit H (AL356013-1)	188.7	92.5	85.5	616.6	917.6	1007.7	157.5

The nucleotide sequences of anther- or pistil-specific genes have been deposited in GenBank, EMBL and DDBJ databases (accession numbers AU240045–AU240182).
[a]A group consisted of cDNA clones which have an overlap sequence over 95% identity for more than 100 bp (see Section 2.6).
[b]The number of cDNA clones within the group.
[c]Gene with functional annotation with maximum homology is obtained using BLASTX program.
[d]Averaged signal intensity of replicated experiments within each group.
[e]No data.

cant homology to known sequences (BLASTX score <100) or were similar to proteins with unidentified functions (data not shown), indicating that the function of about half of the anther-specific clones in both clusters I-a and II-a could not be determined by sequence similarity. These clones are considered to be novel anther- and/or pollen-specific genes, however, the sequence of the entire cDNA would be required to confirm that they are indeed novel.

In order to compare the entire data set for each sample (anthers 1–3, pistils 1–3, and leaves), we constructed a dendrogram (Fig. 1). Anther-2 and anther-3 are the first clusters, and they connect to anther-1. This indicates that the expression pattern of anther-1 should reflect the transcripts derived from microspores and/or tapetum cells, and the expression pattern of the anther-2 and anther-3 should reflect the transcripts derived from the pollen grains. Furthermore, leaves and all stages of pistils combine in a single cluster, indicating that the expression patterns of the anther-specific genes are similar in these four tissues, and the anther is a differentiated organ relative to the leaf and pistil.

3.4. Functional characterization of genes specifically expressed in anther tissues

As described above, approximately half of the genes which were defined as anther-specific genes were annotated to known functional genes.

In the case of cluster I-a, a cDNA clone (group A-76) is homologous to *cer1*, which is involved in epicuticular wax biosynthesis and pollen fertility [22]. In addition, a cDNA clone classified into group A-9 has sequence similarity to a gene encoding a lipid transfer protein (LTP [23]). The expression of *LTP* in *Brassica napus* was observed at the anther microspore stage [23]. The temporal expression of group A-76 and group A-9 is similar to the *cer1* gene and the gene encoding anther-specific LTP, respectively, indicating that these genes would be important in the early developmental stages of anthers in plant species.

A previous study has revealed that soybean cytosolic glutamine synthetase (*GS15*) is specifically expressed in anthers and pollen grains [24]. According to our microarray analysis, a cDNA clone encoding cytosolic glutamine synthetase (group A-204) was predominantly expressed at an early stage of the anther tissues, and was slightly expressed in other organs. Because pollen grains synthesize and store a large amount of proteins for pollen germination and pollen tube growth, the high expression of cytosolic glutamine synthetase in anthers can be explained physiologically by the fact that cytosolic glutamine synthetase activity is necessary for cells with high metabolic activity. A cDNA clone encoding Δ^1-pyrroline-5-carboxylate synthetase (groups A-125 and A-176), which is a rate-limiting enzyme in proline biosynthesis [25], displayed a similar expression pattern to a cDNA clone encoding cytosolic glutamine synthetase (group A-204). Because glutamine rather than glutamate is the major amino acid used for proline synthesis via a constant pool of glutamate [26], the high expression of the cDNA clone encoding glutamine synthetase and Δ^1-pyrroline-5-carboxylate synthetase in anthers suggests that a large amount of proline synthesis occurred in immature anther or pollen. Proline is thought to play an important role as an osmoregulatory solute in plants subjected to drought and salt stress [25]. The high concentration of proline in pollen indicates that proline may serve either as osmotic

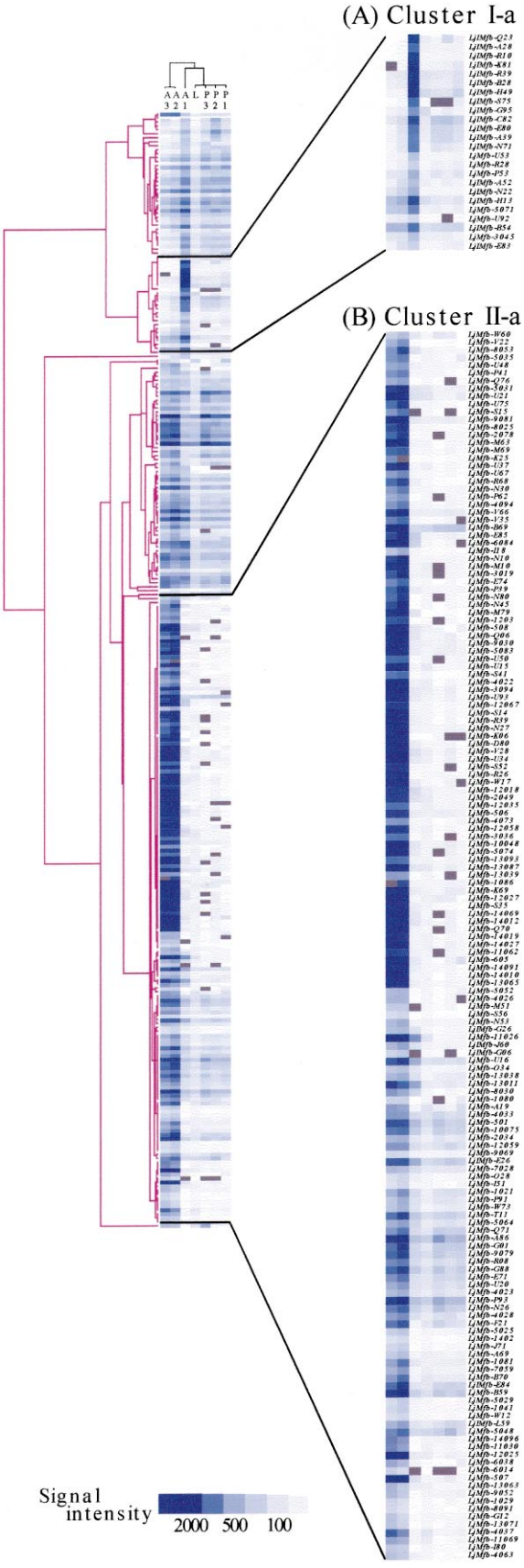
(A) Cluster I-a
(B) Cluster II-a
A A A L P P P
3 2 1 3 2 1
Signal intensity
2000 500 100

Fig. 1. Cluster image of 281 highly expressed cDNAs in the anther. cDNAs were placed in order using average linkage clustering methods to group similar expression patterns. Horizontal and vertical dendrograms indicate the degree of similarity between the expression profile for the cDNA and the developmental stage of organs, respectively. Each column represents the developmental stage of anthers, pistils and leaves. Each row represents the signal intensity of each cDNA clone. In the cluster image, blue indicates high signal intensity and gray indicates missing data. A: Cluster I-a was constructed from cDNA clones specifically or predominantly expressed in anther stage 1, and contained 24 cDNA clones. B: Cluster II-a was constructed from cDNA clones specifically or predominantly expressed in both anther stages 2 and 3, and contained 159 cDNA clones.
←

protection during dehydration or as a nutrient for pollen germination.

Thirteen groups (25 clones) encoded proteins involved in reorganization of the cell wall during cell expansion (e.g. pectin methylesterase, ascorbate oxidase, β-galactosidase, polygalacturonase) in cluster II-a. These proteins have been previously reported to be specifically expressed during late pollen development and to play an active role during pollen germination and pollen tube growth [27–30].

In cluster II-a, a cDNA clone (group A-121) showed the sequence similarity to a gene encoding a pollen allergen (*Ole e* I, [31]), and a cDNA clone (group A-45) showed sequence similarity to a gene encoding acid phosphatase that was identified as a pollen allergenic protein previously [32]. In several plant species, common pollen allergens beyond plant species have been identified [23]. Thus, proteins encoded by these clones also potentially function as pollen allergens in various plants, although their function in plants has not been determined.

Four groups (nine cDNA clones) were identified as genes similar to those encoding proteins related to sugar transport [monosaccharide transporter (group A-93), sucrose transporter (group A-184), glucose transporter (group A-124), and cell wall invertase (group A-81)] in cluster II-a. cDNA clones encoding the monosaccharide and disaccharide transporters, which were specifically expressed in pollen grains, have been isolated in *Petunia* and *Arabidopsis* [33–35]. The genes encoding the sugar transporter form a small multigene family [36].

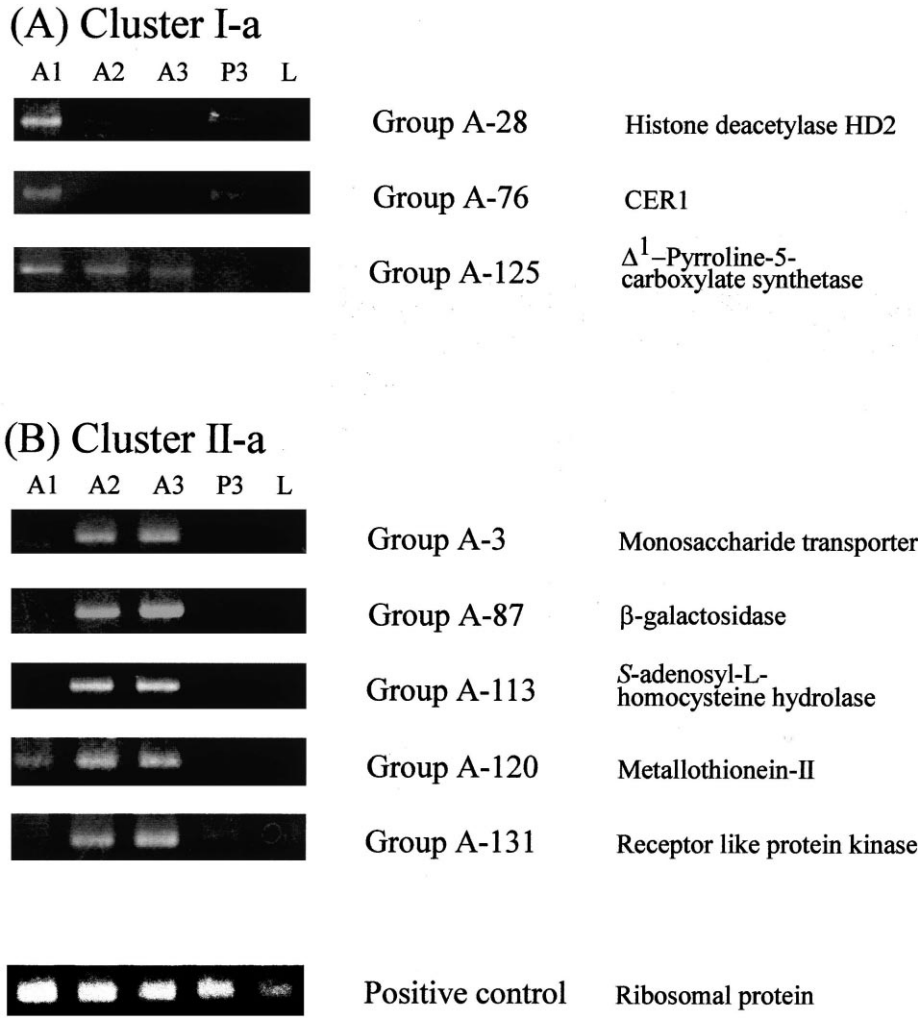

Fig. 2. RT-PCR analysis of anther-specific cDNA clones identified by the cDNA microarray. Poly(A)$^+$ RNA was extracted from anther tissues in stage 1 (A1), stage 2 (A2), and stage 3 (A3), pistil tissues in stage 3 (P3) and from leaf tissues (L). Reverse-transcribed cDNA in each sample was used for PCR amplification with a set of primers *Not*I-dT and a primer specific to each gene. A: Group A-28, group A-76 and group A-125 were derived from cluster I-a. B: Group A-3, group A-87, group A-113, group A-120 and group A-131 were derived from cluster II-a. The gene encoding ribosomal protein was amplified as a positive control.

Thus, plant cells possess sugar transporters tailored to meet their specific requirements at particular tissues or stages of development. In the case of a gene encoding cell wall invertase, which converts the disaccharide to monosaccharide, antisense repression of the gene for cell wall invertase in the anther resulted in a block of pollen development, causing male sterility [37]. Therefore, because it is known that developing pollen grains are strong sinks that require the import of carbohydrates from the apoplast during pollen maturation, pollen germination, and pollen tube growth, genes encoding proteins related to sugar transport, which were identified in this experiment, should function specifically in reproductive organs.

Four groups encoding H^+-ATPase were found in cluster II-a. The plasma membrane H^+-ATPase may be a target of the signaling pathway because it functions in many cellular processes identified in pollen biology, such as the uptake of K^+ [38], and sugars [33,39]. Thus, genes encoding H^+-ATPase specifically expressed in anther may play a significant role in pollen development, germination and growth.

In our microarray analysis, five groups (groups A-12, 25, 128, 144 and 165) encoding actin proteins and one group (group A-93) encoding an actin-depolymerizing factor, which modulates actin organization, were specifically expressed in anther stage 2 and stage 3. The actin cytoskeleton is known to play a key role in the morphogenesis and function of highly specialized cells such as pollen tubes [40]. Actin-depolymerizing factor is known to affect actin organization, cell expansion and growth [41]. In several plant species, genes encoding pollen-specific actin proteins and actin-depolymerizing factors have been reported [42–45]. Thus, genes encoding these proteins also function during the pollen germination and pollen tube growth in *L. japonicus*.

Recently, it was reported that *MZm3-4* encoding metallothionein type II was specifically expressed in the immature tapetum in maize [46]. However, group A-120 encoding metallothionein II was specifically expressed in mature anther in *L. japonicus*. To date, the role of metallothionein II in the anther is not known.

Group A-74 in cluster II-a showed sequence similarity to a gene encoding cytochrome *c*. It has been reported that the transcript levels of cytochrome *c* are high in the anther and pollen in sunflowers [47]. The function of this protein has been implicated in high respiratory activity of mitochondria or in early events of the programmed cell death [48], though the real function of cytochrome *c* in *L. japonicus* cannot be deduced in this experiment.

Other clones, three groups (cluster I-a) and 13 groups (cluster II-a), were annotated to known proteins or peptides (Table 2). Although the tissue specificity of the expression of these clones has not been previously characterized, they are potentially novel anther-specific genes.

3.5. Cluster analysis and functional characterization of cDNA highly expressed in pistil tissues

Cluster analysis of the clones highly expressed in the pistil was performed with the same strategy for identification of anther-specific cDNA clones as described above. We found an interesting cluster (cluster I-p) containing clones which were highly expressed in all stages of pistil development and were expressed at extremely low levels in the anther and leaf tissues. This cluster was constructed from 54 groups (105 clones), and 23 of these 54 groups (46 clones) showed a high degree of similarity to proteins of known functions in a database search analysis.

In these 23 groups, chalcone synthase was encoded by four groups (11 clones), and was known as a key enzyme for the synthesis of phytoalexin [49]. A proteinase inhibitor, which was specifically expressed in stigmatic tissues, has been characterized in *Nicotiana alata* [50]. Two clones (group P-9) encoding the protease inhibitor were also identified. Furthermore, three groups (three cDNA clones) encoding lipoxygenase were identified in our experiment. Lipoxygenase is one of the key enzymes for the biosynthesis of jasmonic acid, a modulator of the plant defense response [51]. Together these genes may function in pathogenic defense, since exudates secreted from stigma and/or style tissues of the pistil provide a favorable environment for the growth of pathogens.

We found cDNA clones (groups P-2 and P-7) encoding LTP in cluster I-p. LTP is proposed to participate in cutin biosynthesis, antimicrobial activity, symbiosis, embryogenesis, and adaptation to various stresses [52]. Recently, it was reported that LTP induced the adhesion of the pollen tube to an artificial stylar matrix in vitro [53]. Although specific expression of the cDNA clones encoding LTP does not provide real evidence of function, it does implicate the important role that LTP may play (pollen adhesion to stylar matrix, antimicrobial peptide, etc.) in the pistil.

In cluster I-p, 31 groups (59 clones) showed no significant sequence similarity to known genes or similarity to proteins with unidentified function (data not shown) and are potentially novel pistil-specific genes.

As described above, anther-specific cDNA clones were classified into two clusters (cluster I-a and II-a). The cDNA clones in each cluster were differentially regulated during each stage of anther development. In contrast, we could not identify cDNA clones that are developmentally regulated in the pistil. The total number of cDNA clones specifically expressed in the pistil was extremely low relative to those in the anther. This is surprising since the pistil is a complex organ which is composed of many specialized tissues (stigma, style, ovule, etc.). Thus, our data may have excluded cDNA derived from these less abundant mRNAs from this complex organ and be skewed towards the genes from the anther, a less complex organ.

3.6. Comparison of the data between cDNA microarray and RT-PCR in anther-specific genes

To evaluate the results obtained by microarray analysis and confirm the reproducibility of our approach, we performed RT-PCR for the 15 cDNA clones and 60 cDNA clones from cluster I-a and cluster II-a, respectively. Representative results of the RT-PCR of eight cDNA clones are indicated in Fig. 2. RT-PCR analysis of candidate cDNA clones showed that 68% (51 genes/75 genes) of the cDNA clones analyzed were differentially regulated as shown by the microarray analysis, indicating that the majority of our microarray data are reliable. This may be attributed to the high number of repetitions (four to five) performed in the microarray analysis.

4. Conclusion

In this study, we identified anther- and pistil-specific genes using a cDNA microarray. Since the size of the reproductive

organs in most plant species is very small and collection of the organs is very laborious, a cDNA microarray is an effective tool for identification of reproductive organ-specific genes. However, to obtain reliable data, a cDNA microarray experiment and RT-PCR experiment should be combined.

We are currently determining the temporal and spatial expression pattern of the cDNA clones identified in this study more precisely using in situ hybridization. Furthermore, to discover the real function of these reproductive organ-specific genes, we will obtain a mutant phenotype of each gene by making transgenic plants with antisense-oriented construct directed by pollen and/or anther specific promoters.

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